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Analysis of ENV V3 Sequences From HIV-1-Infected Brain Indicates Restrained Virus Expression Throughout the Disease

Mariantonietta Di Stefano, Francoise Gray, Thomas Leitner, and Francesca Chiodi

Microbiology and Tumorbiology Center, Karolinska Institute (M.D.S., F.C.), Department of Clinical Virology, Swedish Institute for Infectious Disease Control (T.L.), Stockholm, Sweden; and Department of Neuropathology, Hospital Henri Mondor, Creteil Cedex, and Hospital Raymond Poincaré, Garches Cedex, France (F.G.)

The isolation of human immunodeficiency virus type 1 (HIV-1) from the cerebrospinal fluid (CSF) of asymptomatic virus carriers suggests that the viral infection spreading to the brain occurs early during infection. The aim of the present study was to investigate whether HIV-1 infection of the brain parenchyma also occurs during the early phase of infection. We also wished to compare the degree of replication of the virus in the brain at different clinical stages associated with HIV-1 infection. With the use of polymerase chain reaction (PCR), the viral genomes present in seven of eight brain specimens obtained from two asymptomatic HIV-1 carriers and six AIDS patients were amplified. Thereafter, the number of viral copies present in each brain specimen was quantified, the third variable region (V3) of the gp120 glycoprotein was sequenced and these results compared with the histopathological findings in the tissue.

The HIV-1 DNA genome was amplified from seven of the eight brain tissues, including the specimens obtained from the two asymptomatic carriers. An increased number of viral copies in the brain was found in association with histopathological findings of HIV-1 encephalitis. The analysis of the V3 sequences, however, revealed the presence of a homogeneous virus population in the brain at every clinical stage of the disease. These results suggest that, although entry of the virus in the parenchyma may occur early during infection, HIV-1 replication in the brain is constrained until the terminal phase of AIDS encephalitis. © 1996 Wiley-Liss, Inc.

KEY WORDS: DNA quantification, genetic homogeneity, asymptomatic carriers, sequence analysis, virus genotypes

INTRODUCTION

Studies conducted during the early stage of the HIV-1 epidemic showed the presence of HIV-1 in the brain of AIDS patients [Shaw et al., 1985; Ho et al., 1985]. The brain appeared to be one of the major targets for viral infection and replication when it was compared to other body tissues such as spleen, lymph node and liver [Shaw et al., 1985]. The blood-brain barrier offers a mechanical defense to viral infection of the brain and the question of how HIV-1 invades the central nervous system (CNS) is still open. Entry of free viral particles to the brain may occur during the viremic phase of HIV-1 seroconversion [Gaines et al., 1987]. Moreover, T-cells and macrophages activated by the viral infection may have a preferential ability to cross the blood-brain barrier [Wekerle et al., 1986] as compared to resting macrophages and T-cells, where migration to the brain occurs physiologically through endothelial cell spaces [Hickey, 1991]. Although it is likely that several pathways of the spread of infection may account for the entry of the virus into the brain, HIV-1 infection in this compartment could be facilitated by the meningitis occurring in concomitance with seroconversion to the virus [Cooper et al., 1985].

Several studies have been undertaken to delineate the time period and the frequency of HIV-1 spread to the brain. The results of these studies showed that the invasion of the CNS by this virus is an early and common event during HIV-1 infection. In fact, HIV-1 can be found in the cerebrospinal fluid (CSF) of the majority of asymptomatic viral carriers by conventional isolation techniques on prestimulated mononuclear cells obtained from the peripheral blood of healthy blood donors [Sönnerborg et al., 1988; Chiodi et al., 1992]. The finding that HIV-1-specific IgG and IgM antibodies are present in the CSF of asymptomatic patients [Elovaara et al.,

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Address reprint requests to Francesca Chiodi, Microbiology and Tumorbiology Center, Karolinska Institute, S-17177 Stockholm, Sweden.

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TABLE I. Clinical Stage and Neuropathological Findings of Eight HIV-1 Infected Patients

Patient sex/age	Clinical stage	Neurophatological findings
4501 M/30	Asymptomatic	Few perivascular lymphocytic cuffs in leptomeninges, basal ganglia and white matter.
4624 M/36	Asymptomatic	Few perivascular lymphocytic cuffs in leptomeninges, basal ganglia and white matter
4562 F/30	AIDS	Nondiagnostic changes
4557 M/28	AIDS	Nondiagnostic changes
4516 M37	AIDS	Progressive multifocal leucoencephalopathy
8723 33/M	AIDS	Cerebral lymphoma and chronic cerebral toxoplasmosis
10411 38/M	AIDS	HIV encephalitis and cerebral lymphoma
9594 M/32	AIDS	HIV encephalitis and vacuolar myelopathy; chronic cerebral toxoplasmosis and cerebral lymphoma

1987; Resnick et al., 1988; Marshall et al., 1988; Chiodi et al., 1988] supports further the observation that the spread of HIV-1 into the brain compartment occurs early during infection. Sinclair and Scaravilli [1992] have detected the presence of proviral DNA in the brain of 2 of 8 HIV-1 asymptomatic patients using polymerase chain reaction (PCR). Interestingly, the same authors showed that immunological reactions occurring in the brain during the AIDS phase may be initiated during the asymptomatic phase of infection by an increase in density of microglial cells [Sinclair et al., 1994].

Several questions need to be addressed in order to further our understanding on the kinetics of HIV-1 infection of the brain. It is of interest to ask whether active replication of HIV-1 in the brain occurs at the time of invasion of this compartment, or if viral replication is controlled during the asymptomatic phase of the disease. Moreover, it is unknown whether HIV-1 establishes infection of the brain in one, or several, time points. In order to answer these questions we have quantified the number of viral copies present in eight infected brains by PCR. In addition, we have sequenced the amplified V3 of the env gene PCR products and analyzed the presence of heterogeneous sequence populations in the viral DNA. The results were correlated with histopathological examination of the tissues.

MATERIAL AND METHODS

Subjects and Tissue

The postmortem brain tissues included in the present study were obtained from a series of brains from the Hospitals Henri Mondor and Raymond Poincaré (Table I). Frontal cortex specimens were obtained from the brain of eight adult patients (7 males, and 1 female). According to CDC classification, two of the patients had an asymptomatic HIV-1 infection and six had AIDS. The findings obtained at the histopathological examination of the brains are indicated in table I. Examination of brain tissue obtained from patients 4501 and 4624 revealed only nonspecific neuropathological changes. These include granular ependymitis, mineralization of vein walls and fibrous thickening of leptomeninges and vein walls. Patients 4562 and 4557, who were classified with AIDS, did not show any histopathological changes. Progressive multifocal leucoencephalopathy (PML) was diagnosed in patient 4516; primary CNS lymphoma was found in patients 8723, 10411 and 9594. Histopathological changes consistent with HIV-1 encephalitis and primary CNS lymphoma were diagnosed in patients 10411 and 9594.

HIV-1 was identified in several paraffin-embedded sections from each brain after pretreatment by microwave, using a monoclonal antibody against p24 (Dupont, Paris, France), by an indirect immunoalkaline phosphatase (APAAP) method. Three of the AIDS patients included in the study (4557, 4562, and 4516) had been treated by zidovudine after onset of CD4 decline.

Polymerase Chain Reaction and Determination of DNA Viral Load

DNA extracted from frozen brain tissue was used for PCR amplification. One ml of 4 M guanidium isothiocynate was added to approximately 0.5–1 cm^2 of tissue from each brain specimen. The tissue was homogenized for 2–4 minutes. DNA was extracted with phenol-chloroform (24:1 chloroform:isoamyl alcohol), precipitated with 99% ethanol and then resuspended in distilled water.

The env V3 domain of HIV-1 was amplified by using a nested PCR reaction previously described by Albert and Fenyö [1990]. After the first round of amplification carried out with oligonucleotides JA 9 and JA 12, an additional 30 cycles were conducted with inner primers 11U and the biotinylated 10B [Wahlberg et al., 1991]. This amplification yields a fragment of 350 base pairs that includes both the loop and flanks of the V3 regions of the HIV-1 envelope gene. The DNA amplification products were run on a 1% agarose gel and visualized by ethidium bromide. All PCR amplifications were carried out in duplicate. Quantification of the virus was

undertaken by diluting each DNA sample in H_2O at five 10-fold dilution steps, beginning with a 1:10 dilution, and by testing each dilution in parallel PCR reactions. The first dilution of brain genomic DNA used in each PCR corresponded to 1 μg. Samples containing different concentrations of DNA extracted from HIV-1 chronically infected ACH-2 cells were used as positive controls and were included in each PCR run. Negative controls consisted of specimens containing only PCR reaction mixture and DNA extracted from different types of HIV-1-negative cells.

Direct Sequencing of Viral DNA

The PCR products were sequenced by solid-phase DNA sequencing [Hultman et al., 1991]. The amount of DNA used for each sequencing reaction varied between 3 and 14 pmol. Briefly, the biotinylated PCR fragments were immobilized on streptavidin-coated magnetic beads (Dynal AS, Norway). Strand separation was made by NaOH denaturation and both the immobilized and the supernatant strands were sequenced using T7 DNA polymerase. The sequences of the fluorescin-labeled sequencing primers 10F and U have previously been published [Wahlberg et al., 1991]. Sequences were determined in an automated laser fluorescent (ALF) sequencing apparatus (Pharmacia, Uppsala, Sweden).

In order to evaluate the presence of HIV-1 DNA polymorphisms in the sequenced PCR products, the sequences obtained with the ALF were edited manually. A previous study by Leitner et al. [1993] showed that solid-phase sequencing is an efficient and sensitive method for the analysis of mixed populations in the V3 loop of the env gene of HIV-1.

RESULTS

PCR Amplification and Semiquantification of HIV-1 DNA Present in the Brain

HIV-1 DNA could be found in seven of the eight brain specimens included in this study by PCR with a set of primers which amplified the V3 loop region of the env gene. HIV-1 was detected by this PCR system in the specimens obtained from both the two asymptomatic virus carriers (4501 and 4624) and in five of the six AIDS patients. The amplified V3 loop PCR products from 5 of the 8 brain specimens are shown in Figure 1.

The sensitivity of the nested PCR protocol has been assayed previously using serial dilutions of HIV-1 DNA [Brichman et al., 1991]. We could detect a single target molecule in the presence of a high background of genomic DNA (1 μg). Serial dilutions of the DNA extracted from the seven HIV-1-positive brain specimens were used to quantify the amount of viral DNA copies present in the infected tissue. The number of copies per μg of genomic DNA was between 1 and 10 in the two asymptomatic subjects and in three AIDS patients without signs of HIV-1 encephalitis. A larger number of viral DNA copies was found in the brain of two AIDS patients where histopathological examination revealed primary CNS lymphoma and HIV-1 encephalitis (Table II). In the specimen from patient 10411, the number of viral copies ranged between 10 and 100 and in patient 9594 between 100 and 1,000.

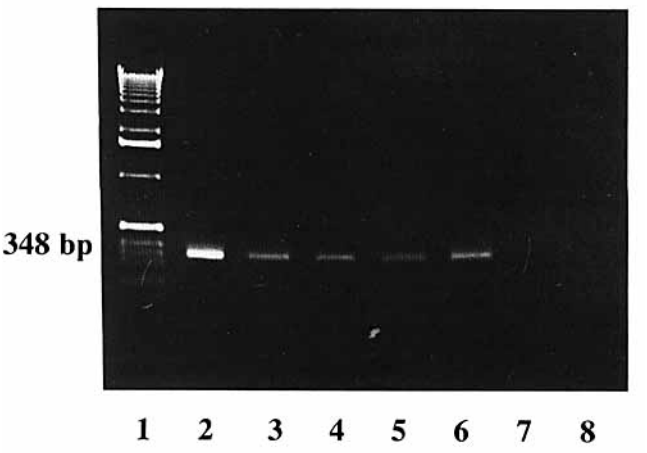

Fig. 1. PCR amplification of viral DNA from brains of HIV-1-infected patients. **Lane 1** shows the DNA marker (1 kb DNA ladder); **lane 2,** DNA extracted from ACH-2 cells; **lanes 3–7,** DNA extracted from patients 4557, 4501, 4516, 4624, 4562; and **lane 8,** DNA extracted from uninfected cells.

TABLE II. Number of HIV-1 Copies per μg/Brain DNA

	Range of DNA copies detected at each PCR dilution			
Patient	1–10	10–10^2	10^2–10^3	10^3–10^4
4501	+	–	–	
4624	+	–	–	
4562	–	–	–	–
4557	+	–	–	–
4516	+	–	–	–
8723	+	–	–	–
10411	–	+	–	–
9594	+	+	+	–

A good correlation was observed between the results obtained by our semiquantitative PCR method and those obtained by immunocytochemistry as reported by others [Achim et al., 1994]. In fact, the presence of p24 antigen was demonstrated in the specimens obtained from patients 10411 and 9594 in which the histopathological examination revealed HIV-1 encephalitis and in which we found high HIV-1 copy numbers. On the contrary, p24 expression could not be revealed in the brains from the remaining patients who had minor histopathological changes and low viral DNA copies.

Analysis of the HIV-1 V3 Sequences Derived From the Infected Brains

We derived the amino acid (aa) sequences of the V3 region by direct sequencing of the PCR products. The phylogenetic analysis (results not shown) of the sequences revealed that all patients were infected with viruses belonging to the genetic subtype B [Myers et al., 1994]. The amino acid sequences are shown in Figure 2, aligned with the consensus sequence of the genetic subtype B of HIV-1. In our samples, between 2 and 10

A

```
4501      TGT ACA AGA CCC AAC AAC AAT ACA AGA AAA GGT ATA
4624      TGT ACA AGA CCC AAC AAC AAT ACA AGA AAA AGT ATA
4557      TGT ACA AGA CCC AAC AAC AAT ACA AGG AAA AGT ATA
4516      TGT ACA AGA CCC AAC AAC AAT ACA AGA AGA AGT ATA
8723      TGT ACA AGA CCC AAC AAC AAT ACC AGG AAA AGT TTA
10411     TGT ACA AGA CCC AAC AAT AAT ACA AGA AGA AGT ATA
9594      TGT ACA AGA CCC AAC AAC AAT ACA ATA ACA GGT ATA

4501      TAT ATA GGA CCA GGC AGA GCA TTT TAT GCA ACA GGA
4624      CAC ATA GGA CCA GGG AGT GCA TTT TAT GCA ACA GGA
4557      AAT ATA GGA CCA GGC AGA GCA TTT TAT ACA ACA GGA
4516      CAC ATA GGA CCA GGG AGA GCA TTG TAT GCA ACA GGA
8723      CAT ATA GGA CCA GGG AGA GCA TTT TAT ACA ACA GCA
10411     TCT ATG GGA CCA GGG AGA GCA TTT TGG GCA ACA AAT
9594      CAT ATA GGA CCA GGC AGA GCA TTT TAT ACA CAA GGA

4501      GAC ATA ATA GGA GAT ATA AGA CAA GCA CAT TGT
4624      GGA ATA ATA GGA GAT ATA AGA CAA GCA CAT TGT
4557      GAA ATA ATA GGA GAT ATA AGA AAA GCA CAT TGT
4516      GGA ATA ATA GGA GAG ATA AGA CAA GCA CAT TGT
8723      GAA ATA ATA GGA GAT ATA AGA CAA GCA CAT TGT
10411     GAT GTA ATA GGA GAT ATA AGA AAA GCA TAT TGT
9594      CAA ATA ACA GGA GAT ATA AGA CGA GCA CAT TGC
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B

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                          *               *
B CONSENSUS*    CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC
4501            ----------Y----S---A--G---- -----
4624            ----------G-Y--------A--D---- -----
4557            ----------N----------E------K---
4516            ---------R---------L-A--G---E------
8723            --- -------L----------------------
10411           ---------R--SM------WA-NDV----K-Y-
9594            ---------ITG----------Q-Q-T----R---
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Figure 2.

aa substitutions were found in comparison to the consensus, regardless of the clinical stage of the disease or the presence of neurological manifestation. The sequence obtained from the brain of patient 8723, who had cerebral lymphoma and chronic cerebral toxoplasmosis, showed a high degree of homology with the consensus sequence (only one substitution) (Fig. 2).

Six of seven sequences had the conserved apical tetrapeptide GPGR [La Rosa et al., 1990], whereas one sequence from the brain of patient 4501 contained a different loop apex, namely, GPGS (Fig. 2). The blood sample sequence from the same patient showed the same tetrapeptide as the brain sample (data not shown).

The nucleotide sequences were edited manually in order to screen for the presence of polymorphic HIV-1 populations in the brain specimens. Since we had quantified the number of HIV-1 DNA copies in the PCR we were able to analyze the populations whether or not they were heterogeneous by searching for polymorphisms in the sequences [Leitner et al., 1993]. In two of the samples with lower than 10 copies, several PCR amplifications were pooled to avoid artifacts due to single copy amplification. The method has been shown to give as accurate estimates of the heterogeneity of complex clinical populations as plasmid cloning and limited dilutions [Albert et al., 1994]. In reconstitution experiments, minor variants present at the percentage of 10% of the population were regularly detected in multiple analysis [Leitner et al., 1993]. The analysis of the sequences did not reveal the presence of mixed viral populations in the specimens obtained from either asymptomatics and AIDS patients without neurological manifestations or AIDS patients with HIV-1 encephalitis.

The V3 Sequences Suggest the Presence of HIV-1 With Nonsyncytia-Inducing Genotype in the Brain

HIV-1 isolates can be distinguished according to their fusogenic activity in vitro: in variants with high fusion activity in PBMC cultures, named syncytia inducing (SI), and virus with low fusion activity, named nonsyncytia inducing (NSI) [Tersmette et al., 1988]. Approximately 50% of AIDS patients carry virus with SI phenotype, whereas NSI are found in the majority of asymptomatic carriers and persist in the other 50% of AIDS patients [Koot et al., 1993]. The third variable (V3) region of HIV-1 has been suggested to determine the HIV-1 phenotype. Fouchier et al. [1992] have shown that positively charged aas at position 11 and/or 25 in the V3 are usually found in variants with high fusion activity. On the contrary, the V3 sequences of NSI variants have negatively, or noncharged aas at the same positions [Fouchier et al., 1992].

As shown in Figure 2, all sequences showed negative and/or noncharged aa residues at positions 11 and 25 of the loop. Five of the sequences had a serine (S) at position 11 of the loop, whereas a glycine (G) was found in the sequences from patients 4624 and 9594. More variability was found at position 25 where two sequences had aspartic acid (D), two glycines (G), two glutamic acids (E), and one glutamine (Q). These results suggested that at least five of the patients had a virus with NSI phenotype in the brain. The remaining two patients (4624 and 9594), in which a glycine was found at position 11, could have been infected with a SI virus, or were in the phase of transition from NSI to SI variants.

DISCUSSION

HIV-1 invades the CNS early and establishes persistent infection in the brain of both children and adults [Elovaara et al., 1995]. Several studies have shown that the majority of HIV-1-infected individuals, regardless of the stage of the disease or the presence of neurological manifestations, carry HIV-1 in the brain [Marshall et al., 1988; Resnick et al., 1988; Chiodi et al., 1992; Sinclair and Scaravilli, 1992]. The presence of HIV-1 in this compartment has been demonstrated by morphological, virological and molecular biological methods. In fact, HIV-1 may be isolated directly from the brain and/or CSF, and viral particles may be identified by using electron microscopy, immunohistochemistry and PCR amplification [Marshall et al., 1988; Sönnerborg et al., 1988; Budka, 1990; Chiodi et al., 1992; Sinclair and Scaravilli, 1992].

In order to study whether HIV-1 replication in the brain increases with deterioration of neurological manifestation of AIDS, we have selected eight brain specimens obtained from HIV-1-infected patients with different clinical stages of the disease, including two immunologically asymptomatic carriers. We evaluated the presence of HIV-1 DNA in the brain specimens, quantified the viral load, derived the amino acid sequences of the V3 loop and evaluated the presence of heterogeneous sequences, which may reflect the presence of multiple viral populations in the tissue.

We detected viral DNA in seven of the eight brain specimens by PCR amplification. Among the positive samples, we also included those obtained from two asymptomatic carriers who died of causes other than HIV-1 infection. The results are in agreement with the findings reported by others who identified the presence of HIV-1 DNA in the brain of approximately two-thirds of analyzed brain specimens [Shaunak et al., 1990; Böni et al., 1993; Achim et al., 1994]. The presence of the virus in the brain parenchyma of two asymptomatic carriers who died prior to the development of immunological and neurological complications of AIDS lends support to the results presented by Sinclair et al. [1994], who detected HIV-1 proviral

Fig. 2. Analysis of the nucleotide **(A)** and amino acid sequences **(B)** of the V3 loop obtained by direct sequencing from seven HIV-1-infected brains. The sequences are aligned against the consensus sequence of HIV-1 subtype B [Myers et al., 1994]. Dashes indicate an amino acid identical to that present in the consensus sequence. Asterisk: amino acid positions involved in determining the viral phenotype according to Fouchier et al. [1992].

DNA in the brains of two of eight HIV-1-positive asymptomatic individuals using the PCR technique. Although several studies have shown the presence of the virus in the CSF of asymptomatic HIV-1 carriers [reviewed in Elovaara et al., 1995], thus suggesting early invasion of the CNS by HIV-1, it was unclear whether the source of origin of the virus was the blood or the brain tissue. Our studies indicate that the virus is present in the brain compartment and suggest that the presence of lymphocytic meningitis, cerebral vasculitis and gliosis of the white matter in asymptomatic patients may be due to immunological responses mounted against the early CNS invasion by the virus [Gray et al., 1992]. The presence of sequences with NSI genotype [Fouchier et al., 1992] in the tissues obtained from the majority of patients suggests that the spread of the virus to the brain may occur during the early stage of the infection.

Power et al. [1994] reported recently an association between two amino acid positions (305 and 329) within the V3 region of HIV-1 strains amplified from brain specimens and the clinical signs of AIDS dementia. This result is not confirmed in the V3 HIV-1 sequences originated in our laboratory from the brain and cerebrospinal fluid [Keys et al., 1993]. The lack of correlation between our findings and the results presented by Power et al. [1994] will be published separately (Di Stefano et al., in press). Consideration should be given to whether the source of viral DNA in the brain specimens can be due to the presence of infected lymphocytes infiltrating this tissue [Bell et al., 1993] or to direct infection of neuronal cells. Whatever the cellular carrier for the virus, the positive PCR amplification of HIV-1 genome from the brain of asymptomatic viral carriers indicates that the virus has already reached the nervous system during the asymptomatic stage.

Most of the infected HIV-1 patients remain unaffected neurologically during the first phase of the disease, even if they harbor the virus in the CNS. The possibility that HIV-1 establishes a latent infection of the brain with a low replicative activity in this compartment might explain this phenomenon. In order to study whether the number of viral copies in the brain increases upon disease progression, we quantified the number of HIV-1 copies in seven brain specimens which were PCR-positive. The highest levels of viral DNA were quantified from the two brains with histopathological signs of HIV-1 encephalitis, in which the levels of infected cells were between 1:100 and 1:1,000. The number of copies of HIV-1 DNA in the brain of two asymptomatic and three AIDS patients without neurological changes was generally low (corresponding to 1 infected cell in 10,000). The results obtained by semiquantitative PCR analysis were in agreement with the immunohistological findings which showed high levels of p24 antigen in the brain tissues of the two AIDS patients with HIV-1 encephalitis and was negative in the remaining sample. Our findings are in agreement with earlier studies [Sinclair and Scaravilli, 1992; Böni et al., 1993; Achim et al., 1994], which showed a good correlation between the presence of high level of viral DNA in the brain and histopathologic evidence of HIV-1 encephalitis.

These findings, taken together, suggest an increased rate of viral replication in the brain of patients with histopathological signs of HIV-1 encephalitis. This result is expected in view of the fact that an increased viral load in both blood and lymphoid tissue correlates with depletion of CD4 lymphocytes and disease progression during AIDS [Connor et al., 1993; Pantaleo et al., 1993]. In order to determine if the increase of viral replication in the brain was accompanied by the appearance of viral genetic polymorphism, we sequenced directly the V3 loop of the env gene, a region which has been shown to be an important determinant for neutralization, viral replication, tropism and cytopathogenicity [Cheng-Majer et al., 1990; Javaherian et al., 1989; Fouchier et al., 1992].

The aa analysis of the V3 loop sequence did not reveal the presence of heterogeneous viral populations in any of the sequences obtained from the brain tissues with different histopathological findings. These results suggest that although entry of the virus occurs early during infection, replication is occurring only at the late stage. In fact, HIV-1 variability is generated upon viral replication by the lack of proofreading of the virus enzyme reverse transcriptase. Several studies conducted by sequencing viral DNA and RNA from the peripheral blood have shown that during the first period of infection, patients harbor a homogeneous viral population that diversifies over time and according to disease progression to a more heterogeneous population [McNeraney et al., 1992; Wolfs et al., 1992].

The very homogeneous viral population in the brain could be found also in the presence of severe histopathological findings, thus suggesting that although there is an increase during the late stage of the disease, viral replication in the brain is restrained at any time point. Korber et al. [1994] also observed HIV-1 sequence homogeneity when comparing HIV-1 cloned sequences obtained from the blood and brain of HIV-1-infected patients with severe neurological signs and symptoms. The lack of viral polymorphism in our specimens also suggests that multiple infectious events do not occur frequently in the brain compartment. This hypothesis is supported by the findings of Bockstahler et al. [1995] who have indicated that primary HIV-1 infection takes place at a restricted area of the brain. This initial site of infection represents the source for viral spread to other regions of the brain.

In conclusion, our studies indicate that although an increase in viral replication can be noticed in association with severe histopathological lesions in the brain of HIV-1-infected individuals, viral replication is restrained in this compartment. The pathogenesis of AIDS-related neurological syndromes and histopathological changes in the brain is not clear. Our findings suggest that if the virus is directly involved in causing changes in the brain which lead to the clinical symptoms, a small amount of the virus or viral factors are sufficient to affect brain function during AIDS.

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